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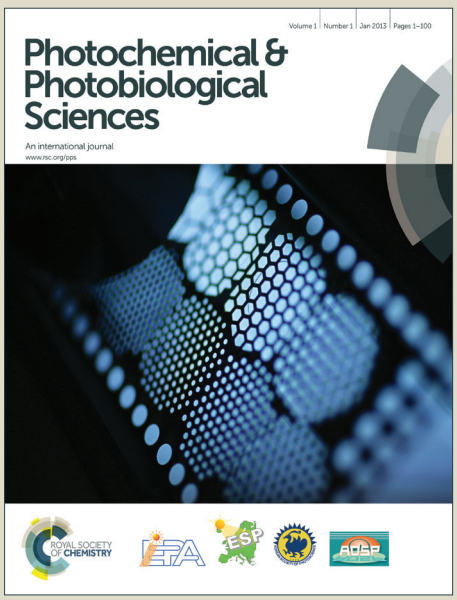

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Photobiostimulation reduces edema formation induced in mice by Lys-49 phospholipases A_2 isolated from *Bothrops moojeni* venom.

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Abstract

The prominent local myotoxic effects induced by *Bothrops* snake venom are due, in part, to myotoxins. This effect is not neutralized by antivenom which is the main therapy to treat victims of snakebite. Two basic myotoxins named MjTX-I and MjTX-II, were isolated from *Bothrops moojeni* venom. Both myotoxins have a Lys-49 phospholipase A_2 structure devoid of enzymatic activity, but are highly myonecrotic and edema-inducing. In this study, we analyzed the effect of low-level laser (LLL) 685 nm, energy density of 2.2 J/cm^2, irradiation time of 15 s, and light emitting diode (LED) 635 or 945 nm at energy density of 4 and 3.8 J/cm^2, irradiation time of 41 and 38 s, respectively, applied 30 min and 3 hs after edema formation in mice caused by MjTX-I or MjTX-II. MjTX-I or MjTX-II caused a significant edema formation, in envenomed paw. LLL and LEDs irradiation significantly reduced the edema formation by both myotoxins from 1 up to 6 hours after the injection. Both LLL and LEDs were similar in reducing the edema formation induced by myotoxins. The combined photobiostimulation with antivenom had the same effect in reducing edema that treatment with the LLL or LEDs alone. In conclusion, the results of this study indicate that photobiostimulation could be used in association with antivenom therapy for treatment of local effects of *Bothrops* species venom.

Introduction

More than 90% of the snakebites reported every year in Latin America are inflicted by snakes of the genus *Bothrops*. [1, 2] The envenomations caused by Bothrops genus are characterized by important local tissue damage, with prominent edema that appears rapidly after the bite at the site where the venom was injected[3]. Such edema, in turn, contributes to hypovolemia and may promote increments in intracompartmental pressures in some muscle compartments, thus inducing further ischemia and tissue damage[4, 5] Moreover, the local effects are very important in terms of medical and scientific interest since the antivenom administration does not efficiently neutralize the proteins responsible for the local tissue damage process which may lead to permanent tissue loss, disability and, in some cases may require the amputation of the victim's affected limb. 6, 7

The most abundant myotoxic components in *Bothrops* snake venoms correspond to phospholipases A_2 (PLA_2) of classes II, which share structural features with the PLA2s present in inflammatory exudates in mammals.[8, 9] Class II PLA_2s can be further subdivided into two main types commonly referred to as Asp49, which have an Asp residue at position 49, and Lys49 showing a Lys residue at position 49. Different from Asp49 PLA_2s, Lys49 PLA_2s have little or no enzymatic activity.[10] In addition to their primary catalytic function, snake venom PLA_2s show a variety of toxic/pharmacological effects which include neurotoxic, myonecrotic, cardiotoxic, hemolytic, hemorrhagic, hypotensive, anticoagulant, platelet aggregation inhibition and proinflammatory activities such as edema formation and leukocyte influx .[11,12]

Bothrops moojeni is a South American snake that causes a number of envenomations in Southeastern Brazil. Two basic myotoxins of approximately

13.5 kDa, named MjTX-I and MjTX-II, were isolated from *Bothrops moojeni* venom. [13-15] MjTX-I and -II do not show catalytic activity on artificial substrates as well as clotting or hemorrhagic activities, like others Lys 49 PLA_2, but are highly myonecrotic and edema-inducing. [16]

Treatment of snakebites is still carried out using traditional antivenom (AV) therapy. [17] This treatment is effective in reversing the systemic effects caused by the venom. However, snake antivenom therapy is usually unable to prevent the progress of local effects.[17] Thus, there is an important search to find therapies that can complement antivenoms in the neutralization of local tissue damage.

Photobiostimulation with low level laser (LLL) and light emitting diode (LED) have been proposed to complement the treatment of local effects caused by bothropic venom. This proposal is based on the literature that shows a reduction of local effects induced by various *Bothrops* snake venom after photobiostimulation such as myonecrosis[18, 19]; inflammation[20-22] hemorrhage[22] and pain.[21, 23] Moreover, Barbosa *et al*., (2010)[24] reported that LLL treatment significantly reduced muscle edema, leukocyte influx and myonecrosis induced by BthTX-I or -II isolated from *B. jararacussu* snake venom.

The present study was therefore designed to evaluate the effects of LLL and LED treatment on the outcome of edema formation induced by MjTX-I or MjTX-II. Moreover, the effectiveness of available *Bothrops* AV used alone or in combination with LLL or LED treatment was also evaluated.

Materials and methods

Animals

All animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). The experiments were performed using 45-day-old male Swiss mice (22–25 g) that were kept in plastic cages with water and food *ad libitum* and maintained under a controlled temperature on a 12-h light/dark cycle. Mice were divided into experimental groups of five animals each.

Myotoxin I (MjTX-I) and Myotoxin II (MjTX-II)

B. moojeni myotoxins I and II were purified as previously described[15, 16], and were kindly provided by Oswaldo Cruz Foundation, Federal University of Rondonia, 76812-245, Porto Velho, RO, Brazil

Evaluation of paw edema

MjTX-I or MjTX-II solution was prepared by diluting 10 µg of myotoxin in 50 µL of a sterile saline solution that was injected intradermally into the subplantar region of the right hind paw. The left hind paw received an equal volume of sterile saline (50 µL) and served as control. The volumes of both hind paws were measured by plethysmometry (plethysmometer model 7140; Ugo Basile, Comerio, Italy) before and 15, 30 min, 1, 2, 3 and 6 h after venom administration according to the method described by Van Arman *et al.* (1965)[25]. Edema was expressed as the percentage increase in volume of the treated (right) paw relative to that of the control (left) paw at each time point.

Light sources, doses and treatment

A low-level semiconductor GaAs laser (LLL; Theralaser D.M.C.; São Carlos, SP, Brazil) and two LED device, one on the red region and one on the infrared region (model Super Red LED-RL5-R3545; Super Bright LEDs, St. Louis, MO) were used for irradiation. The optical power of the laser was calibrated using a Newport multifunction optical meter (model 1835 C). The experimental parameters for the LLL and LED are presented in Table 1. The LLL and LED doses, low enough to avoid any thermal effect, were chosen on the basis of previous studies in our laboratory[20, 22] that had shown a beneficial effect of LLL and LED on the local effects induced by Bothropic venoms. The animals were immobilized manually and the LLL or LED irradiation was applied to the same area as the injection of MjTX-I or -II or saline solution. The control group was treated using the same experimental procedure but with the laser or LED turned off. The animals were irradiated 30 min and 3 h after intraplantar myotoxin injection to evaluate the edema formation.

To investigate the effects of photobiostimulation combined with AV treatment, a volume of 50 µl of AV was injected intravenously 30 min after intraplantar injection of MjTX-I or -II. Animals were irradiated with LLL or LED, and oedema was evaluated as described above. The volume of AV used was sufficient to neutralize the amount of *bothrops* venom injected since 1 mL of antivenom neutralizes 5 mg of *Bothrops* venom (according to manufacturer's specifications).

Statistical analysis

Results are presented as mean ± standard error of mean. Statistical evaluation of data was carried out by one-way analysis of variance (ANOVA) and sequential

differences among means were analyzed by Tukey test (Instat 3.01, GraphPad Software Inc, USA). Differences of results were considered statistically significant when $p < 0.05$.

Results

Edema formation induced by MjTX-I and -II.

Intraplantar injection of 10 μg of MjTX-I or MjTX-II into the mouse hind-paw caused time-dependent increase in paw volume (edema). The maximum hind-paw swelling occurred 1 h after both myotoxins injection. However, MjTX-II caused an increase in paw volume at 1 h that was 44% higher than that caused by MjTX-I (Fig. 1).

Effect of LLL on edema formation induced by either MjTX-I or -II.

Treatment with LLL caused a reduction of 60 and 50 % on edema formation induced by MjTX-I at 1 and 3 h, respectively (Fig. 2A). Likewise, LLL caused a reduction on edema formation which was 69.2, 45.5 and 25% lower than that caused by MjTX-II at 1, 3 and 6 h, respectively (Fig. 2B).

Effect of red and infrared LED on edema formation induced by either MjTX-I or -II.

As demonstrated in Figure 3, irradiation with either red or infrared LED significantly reduced MjTX-I (Fig. 3A) and MjTX-II (Fig. 3B)-induced paw edema. Both LED irradiation significantly reduced paw edema from 1 up to 6 h, in the same magnitude.

Effect of LLL combined with AV on edema formation induced by either MjTX-I or -II.

Treatment with AV 30 min after MjTX-I or MjTX-II injection did not reduce edema formation (Fig. 4 and 5). The combination of AV treatment with LLL irradiation after MjTX-I injection reversed the observed edema from the 1st up to 6 h of evaluation (Fig. 4A). Similar results were observed for MjTX-II (Fig. 4B).

Effect of LED combined with AV on edema formation induced by either MjTX-I or -II.

Concomitant treatment of AV with red LED was effective in inhibiting edema formation of mice induced by MjTX-I similarly to that observed when animals were treated with the red LED alone (Fig. 5A). Also, for the infrared LED, once again, no changes on edema formation were observed when MjTX-II was injected and both treatment were used (Fig. 5B). Both wavelengths caused inhibition of edema formation of the same magnitude.

Discussion

The rapid development of the local effects caused by botropic venom together with the incapacity of antivenoms to neutralize them, frequently result in the appearance of permanent physical and psychological sequelae in patients.[5, 26] For these reason the search for new alternative treatments to the local effects induce by *Bothrops* snakebite, simultaneous with antivenom therapy, is imperative. In the current study we investigated the capacity of photobiostimulation using LLL and LED device to decrease the edematogenic response induced by myotoxins MjTX-I and II isolated from *B. moojeni* venom. Both myotoxins are Lys49-PLA_2, i.e. myotoxins with practically no hydrolytic activity on artificial substrates.[27] However, they induce myonecrosis and edema that are not neutralized by conventional antivenom. [12]

In an attempt to investigate the efficacy of phtobiostimulation on the outcome of edema formation induced by myotoxins, either LLL at 685 nm or LED on two wavelengths, at 635 or 945 nm were carried out. LLL and LEDs were applied 30 min after injection of myotoxins, at the same area. This time of laser or LED applications was chosen based in a previous work from our laboratory that shown a reduction of edema formation and hemorrhage after *Bothrops moojeni* venom injection.[22] In the present work, LLL and both LED irradiation caused a significant edema reduction, when applied 30 min and 3 h after MjTX-I or II injections. Similar results were found in the literature, which shows that LLL at a dose of 4.2 J/cm^2 was capable of inhibiting inflammatory process (edema and leukocyte influx) and myonecrotic process induced by BthTX-I a Lys-49 and BthTX-II an Asp-49 myotoxins isolated by *B. jararacussu* venom.[24] These authors also showed that LLL acts at the same intensity to reduce the inflammatory and myonecrosis processes for both BthTX-I and BthTX-II suggesting

that enzymatic activity is not relevant for laser treatment. Furthermore, literature shows that laser or LED irradiation caused a reduction of muscle edema formation induced by crude venom of *B. jararacussu*[20] and paw edema induced by *B. jararacussu* and *B. moojeni* venom.[21, 22] The mechanism by which photobiostimulation reduces venom-induce edema is not known. Experimental studies have demonstrated that venom PLA_2-induced paw edema depends on the release of histamine and 5HT by mast cell degranulation,[28] also further vasoactive substances such as prostaglandins and kinins could mediate the local edema formation in response to snake venom PLA_2.[29, 30] Moreover, Galvão Nascimento *et al*., (2010)[31] have shown that the edema formation induced by *B. moojeni* venom is mediated by mast cell degranulation, prostaglandins and leukotrienes. In our experimental model, we suggest that the photobiostimulation acted through a reduction of mast cell degranulation and in the release of prostaglandins, leukotrienes and kinins levels. Several studies have shown a reduction of prostaglandins by inhibition of COX-2 expression[32, 33] and kinins[34] after LLL irradiation, providing support to our hypothesis.

The capacity of antivenom to neutralize the venom-induced paw edema induced by myotoxins was also presently investigated. Our results demonstrated that treatment with AV 30 min after MjTX-I or MjTX-II myotoxins inoculation was not able to reduce paw edema formation. These results agree with the literature that shows the inability of AV to neutralize local effects caused by various Bothropic venom and isolated myotoxins. [6, 20, 27]

In further studies, we evaluated the combined photobiostimulation with AV treatment; we found that photobiostimulation associated with AV treatment had the same effect on edema as LLL or LED irradiation alone. Similar results were showed by Nadur-Andrade et al., (2011)[22] where photobiostimulation combined with AV was

effective in reducing edema and hemorrhage induced by *Bothrops moojeni* venom in the same magnitude to photobiostimulation alone. In contrast to our findings, a previous study with laser irradiation associated with AV treatment produced the greatest reduction in muscle edema, after 24 hs of *B. jararacussu* venom injection, when compared with each treatment separately.[20] This diffetence could be be due to the rapid development of the paw edema in contrast to muscle edema.

In conclusion, this work indicates that photobioestimulation is able of inhibiting edema caused by PLA_2 myotoxins. Moreover, the fact that LLL and LED can penetrate into the skeletal muscle could allow non-invasive treatment to be carried out with a low likelihood of treatment-related adverse events. Furthermore, photobioestimulation with the parameters used herein should be considered as a potential therapeutic approach for the treatment of local effects of *Bothrops* snakebite as well as an interesting tool for the study of the mechanisms underlying the inflammatory process activity induced by those myotoxins.

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This research was approved by the Ethics Committee for Animal Research of the Universidade Nove de Julho, SP, Brazil (UNINOVE) under protocol number AN0021/2011.

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Table 1- Protocol for LLL and LED irradiation

Parameters	LLL	Infrared LED	Red LED
Energy density (J/cm^2)	2.2	4	3.8
Power (mW)	30	110	120
Irradiation time (s)	15	41	38
Irradiated area (cm^2)	0.2	1.2	1.2
Wavelength (nm)	685	635	945
Energy per point (J)	0.45	4.51	4.56

Legends

Figure 1- Time course of mouse paw edema induced by MjTX-I or II. Increase in paw volume was determined at selected periods of time after the i.pl. injection of MjTX-I or -II (10 μg/paw) into one paw and apyrogenic saline into the contralateral paw (control paw). The volume increase of paws (edema) was measured by plethysmometry. Data are expressed as % of change as compared to control paws. Each point is the mean ± SEM of 5 animals. *$p < 0.05$ compared with MjTX-I.

Figure 2- Effects of LLL on paw edema formation induced by MjTX-I or -II. MjTX-I (**A**) and MjTX-II (**B**). Mice were injected with 10 μg/paw of either MjTX-I or -II in the right paw. Laser irradiation was performed 30 min and 3 h after myotoxins injection (arrow). The volume increase of the paws (edema) was measured by plethysmometry. Data are expressed as % of change as compared to control paws. Each point represents the mean ± SEM of 5 animals. *$p<0.05$ compared with MjTX-I or II without irradiation.

Figure 3- Effects of red and infrared LED on paw edema formation induced MjTX-I (**A**) or MjTX-II (**B**). Mice were injected with 10 μg/paw of either MjTX-I or -II in the right paw. LED irradiation was performed 30 min and 3 h after myotoxins injection (arrow). The volume increase of the paws (edema) was measured by plethysmometry. Data are expressed as % of change as compared to control paws. Each point represents the mean ± SEM of 5 animals. *$p<0.05$ compared with MjTX-I or II without irradiation.

Figure 4- Effects of LLL and AV treatment on edema formation induced by MjTX-I (**A**) or MjTX-II (**B**). Mice were injected with 10 μg/paw of either MjTX-I or -II in the right paw. The volume increase of the paws (edema) was measured by plethysmometry. LLL irradiation was applied 30 min and 3 h after mytoxins injection (arrow). AV was administered intravenously 30 min after myotoxins injection. Data are expressed as % of change as compared to control paws. Each point represents the mean ± SEM of 5 animals. *p< 0.05 compared with MjTX-I or -II without irradiation.

Figure 5 - Effects of red LED or infrared LED and AV treatment on edema formation induced by MjTX-I (**A**) or MjTX-II (**B**). Mice were injected with 10 μg/paw of either MjTX-I or -II in the right paw. The volume increase of the paws (edema) was measured by plethysmometry. LED irradiation was applied 30 min and 3 h after mytoxins injection (arrow). AV was administered intravenously 30 min after myotoxins injection. Data are expressed as % of change as compared to control paws. Each point represents the mean ± SEM of 5 animals. *p< 0.05 compared with MjTX-I or -II without irradiation.